

**IPSCS FROM PEOPLE WITH MS CAN DIFFERENTIATE INTO  
OLIGODENDROCYTES IN A HOMEOSTATIC BUT NOT AN  
INFLAMMATORY MILIEU**

by

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# Abstract

Multiple sclerosis (MS) is an inflammatory and demyelinating disease of the central nervous system (CNS) that results in variable severities of neurodegeneration. The understanding of MS has been limited by the inaccessibility of the affected cells and the lengthy timeframe of disease development. However, recent advances in stem cell technology have facilitated the bypassing of some of these challenges. Towards gaining a greater understanding of the innate potential of stem cells from people with varying degrees of disability, we generated induced pluripotent stem cells (iPSCs) from peripheral blood mononuclear cells derived from stable and progressive MS patients, and then further differentiated them into oligodendrocyte (OL) lineage cells. We analyzed differentiation under both homeostatic and inflammatory conditions via sustained exposure to low-dose interferon gamma (IFN $\gamma$ ), a prominent cytokine in MS. We found that all iPSC lines differentiated into mature myelinating OLs, but chronic exposure to IFN $\gamma$  dramatically inhibited differentiation in both MS groups, particularly if exposure

was initiated during the pre-progenitor stage. Low-dose IFN $\gamma$  was not toxic but led to an early upregulation of interferon response genes in OPCs followed by an apparent redirection in lineage commitment from OL to a neuron-like phenotype in a significant portion of the treated cells. Our results reveal that a chronic low-grade inflammatory environment may have profound effects on the efficacy of regenerative therapies.

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# Dedication

This dissertation is dedicated to the memory of Mario Morales Pantoja, John Sherrill Houser, and David Laporte Hunter.

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# Introduction

## 1.1 Multiple Sclerosis (MS)

MS is a chronic autoimmune and inflammatory disease that affects the central nervous system (CNS). The disease is characterized by an autoimmune response directed to myelin, leading to demyelination and disruption of the conduction of electrical impulses. Myelin loss ultimately results in axonal degeneration, mitochondrial stress and neuronal cell death <sup>1,2</sup>. It is the most common cause of progressive neurological disability in young adults <sup>3,4</sup> and it is estimated that in the US alone nearly one million people suffer from MS, affecting more women than men with a gender prevalence ratio of 3:1 <sup>5</sup>. There is no single cause identified as the disease trigger, however there is evidence suggesting that genetic and environmental factors contribute to the risk of disease development.

Genetic risk factors include gene variants associated with immune responses, such as DRB1\*1501 haplotype. Environmental factors include low vitamin D, obesity, smoking, and infection by Epstein-Barr virus<sup>4,6</sup>. The disease course, outcome, and the response to therapies are highly variable and unpredictable<sup>7,8</sup>.

MS is categorized into four types: Clinically Isolated Syndrome (CIS), Relapsing-remitting (RRMS), Secondary Progressive (SPMS), and Primary Progressive (PPMS). However, it predominately falls into RRMS and SPMS. CIS is the first occurrence of neurological manifestations caused by demyelination or inflammation that last at least 24 hours, and it is estimated that more than 85% of individuals would progress to RRMS<sup>9,10</sup>. This is the most common form of MS and is characterized by attacks (relapses) with clear and defined neurological symptoms followed by periods of complete or partial recovery (remission). Most patients will transition to a SPMS course, where there is a worsening of neurological function over time. Approximately 10% of patients are affected by PPMS, where symptoms continue to worsen from the onset of disease with no periods of remission<sup>3,4,11</sup>. While the etiology remains elusive, several disease mechanisms have been identified.

The autoimmune attack against myelin is mediated mainly by autoreactive CD4+ and CD8+ T cells. B cells play a central role serving as primary antigen-presenting cells and potentiate the attack by the secretion of autoreactive

antibodies. In fact, in SPMS, there is evidence of B-cell aggregates, or tertiary lymphoid tissue, found in the meninges. This fortifies the hypothesis that even when MS might start in the periphery, it ultimately establishes strategic sites of B and T-cell differentiation and maturation inside the CNS<sup>12,13</sup>. This CNS's compartmentalized response includes the activation of microglia effectively mounting both peripheral and central immune responses<sup>14</sup>.

Oligodendrocytes, the myelin producing cells, have a key role in disease and recovery. The process of remyelination depends on the ability of oligodendrocyte progenitor cells (OPCs) to migrate to sites of injury, proliferate, and mature to regenerate myelin and provide axonal integrity<sup>15</sup>. While OPCs are present throughout the CNS including areas of demyelination, it is thought that about 20% of people with MS remyelinate to some extent, though for most MS patients this process is hindered. Remyelination is more effective earlier in disease than later, which is when failure to remyelinate becomes major contributor to disability<sup>16</sup>. The reasons behind this failure are unknown, therefore it is important to investigate OPCs under both homeostasis and disease states.

## 1.2 iPSCs to study MS

Despite the fact that MS was characterized more than a century ago we still do not know what causes it and why remyelination fails. Our understanding of the disease has been limited in part by the lengthy time of disease progression. Another restriction to its study is the inaccessibility of the cells of interest. Most of our understanding of disease mechanisms come from animal models, *postmortem* studies, and more recently, imaging techniques such as magnetic resonance imaging (MRI). Animal models, particularly murine models, have been critical for elucidating mechanisms of disease and have been paramount in advancing drug development<sup>11,17</sup>. While much of this success stems from highly conserved similarities between rodents and humans such as origin and migration patterns of OPCs, expression of certain transcription factors, patterns of myelination, and myelin proteins, there are also interspecies differences<sup>18</sup>.

Recent advances in stem cell technology allow for the generation of OPCs and OLs directly from MS patients as they live through the disease, bypassing the restrictions of fetal and *postmortem* studies, and interspecies differences that stem from animal models.

iPSC-derived OPCs from MS patients allow for the study of their basic biology and/or inherent susceptibilities, and provide a window into how these susceptibilities may reflect in the ability of iPSCs to fully differentiate. We can also model specific aspects of MS pathology and study how OPCs respond to those conditions, enhancing our ability to understand disease progression. A distinct advantage of using human iPSC-derived OPC/OL is the ability to bring personalized medicine to the patient.

## 1.3 Thesis Statement

Two of the many challenges in MS are the unpredictable outcomes of neurodegeneration and failure to remyelinate. It is still unknown why some people remain stable through the course of the disease, while others quickly progress to disability. To gain a better understanding, we studied the ability of iPSCs derived from MS patients with varying degrees of disability to fully differentiate to oligodendrocytes under both homeostasis and inflammatory states.



# iPSCs from people with MS can differentiate into oligodendrocytes in a homeostatic state but not an inflammatory milieu

## 2.1 Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) that has become the most common cause of progressive neurological disability in young adults. While the etiology of this disease remains unknown, data suggest that it likely involves a combination of genetic, immunological, and environmental factors, all of which may influence pathology, symptomatic presentation, and disease course and outcome<sup>3</sup>. Despite having been characterized more than 100 years ago, its pathophysiology remains elusive<sup>7</sup>, and both the clinical course and disease outcome are highly variable<sup>4,8</sup>.

Many disease-modifying therapies have been developed to combat disability, some of which have significantly improved the course of MS, but

have thus far been unable to stop or prevent neurodegeneration in its progressive phase<sup>19</sup>. One feature of MS that has recently come into focus for new therapeutics is the potential to repair demyelination. Despite the fact that the adult CNS has an available pool of oligodendrocyte progenitor cells (OPCs), and a single oligodendrocyte (OL) has the capacity to produce 40 myelin segments<sup>20</sup>, remyelination in patients with MS is still incomplete. One study showed that only approximately 20% of patients are thought to remyelinate to some extent<sup>8</sup>, but the mechanisms separating successful and failed remyelination are not well known<sup>16</sup>, even when the progenitors of myelin-producing cells are present at the sites of injury<sup>15,21</sup>.

For OPCs to contribute to remyelination, they likely must migrate to the sites of injury, proliferate, and differentiate into OLs<sup>22</sup>. Each of these processes can be inhibited by cytokines (e.g., IL-6, IL-17, osteopontin, IFN $\gamma$ , TNF $\alpha$ ), chemokines (e.g., CXCL1, CXCL2, CXCL10, CXCL11), cytolytic proteins (e.g., lymphotoxin-a and perforin), and signaling factors (e.g., astrocyte-derived endothelin-1 (ET-1), all of which are known to be present at demyelinated areas<sup>23–28</sup>, thereby establishing a potentially challenging environment for repair. MS is notably heterogeneous but can be broadly categorized into two subtypes, relapsing remitting and progressive MS, depending on the presentation and course<sup>3,4</sup>. Within both subtypes is a wide range of disease severity, with some people exhibiting a stable course with limited or no disability, while others quickly decline, often with rapid

accumulation of severe disability. The mechanisms responsible for these varying outcomes are unclear, and whether there are innate differences in the ability to repair remain to be fully understood.

We thus sought to evaluate the potential of iPSCs from people with varying degrees of disability to differentiate into OLs, and to determine mechanisms that might contribute to failure to differentiate into myelinating oligodendrocytes. Thus, we established a platform to generate and investigate OL lineage cells by employing a relatively recent stem cell technology that allows de-differentiation of peripheral blood mononuclear cells (PBMCs) from adult donors into iPSCs which can then be followed by differentiation into multiple cells of interest, including OPCs<sup>29–32</sup>. Using this system, cells from multiple patients can be simultaneously generated. The ability to study human cells, particularly from people with disease, adds an important element to the study of MS. While much information on OPC biology and differentiation has been generated in rodent developmental systems that provide a framework for complementary human studies, some critical notable differences may be relevant to human disease. For example, most rodent studies utilize OPCs from neonatal animals during a developmental state, and OPCs from adults exhibit at least some different properties<sup>33–35</sup>.

Rodent cells differentiate in a matter of days, while human cells require months in culture, thereby allowing a time scale that is much more reflective of humans and allows chronic exposure to varying conditions. Further, rodent cells are typically obtained from healthy pups, which may not be reflective of disease states, and are from homogeneous donors. We thus developed an in vitro platform in which iPSCs were generated from PBMCs of people with either stable MS with low disability score or progressive MS with high disability score. These cells were then differentiated into mature OLs.

During the course of culture, the cells were analyzed both in a non-inflammatory/homeostatic state and in an inflammatory environment to better reflect a disease milieu. Specifically, to model an inflammatory environment, we exposed developing OPCs to interferon gamma (IFN $\gamma$ ), which is a cytokine of particularly high relevance in MS. IFN $\gamma$  is secreted by T cells, natural killer cells, and myeloid lineage cells and is often present in MS lesions<sup>36,37</sup>. Rodent studies in both in vivo and in vitro settings have demonstrated that in addition to its pro-inflammatory properties, IFN $\gamma$  also inhibited the differentiation of OPCs into mature OLs, caused cell death through multiple pathways<sup>38–40</sup>, and affected remyelination through endoplasmic reticulum stress<sup>41</sup>. The net result of these combined effects likely promotes a hostile environment for remyelination.

In support of these hypotheses, the results of our present studies show that iPSCs from both stable with low disability and progressive with high disability MS patients were able to differentiate into mature, myelinating OLs but that chronic exposure to low dose IFN $\gamma$  early in development had a profound negative effect on differentiation. These results demonstrate the ability of iPSCs from heterogeneous states of disease to differentiate into OLs in the absence of inflammation. Our findings also illustrate the implications of long-term exposure to even very low concentrations of IFN $\gamma$ , and highlight the importance of evaluating both cytoprotective and remyelination-promoting agents in the context of an inflammatory microenvironment that may influence repair in people with MS.

## 2.2 Methods

### **Patients and cells:**

PBMCs were obtained from donors with full informed consent and under an Institutional Review Board protocol and institutional Stem Cell Oversight committee approved protocols. Patients were evaluated in the MS Clinic and categorized for disease severity based on their expanded disability status scale (EDSS) score and disease duration. Patient pairs were selected to match for age, sex, and duration of disease greater than 20 years but to be discordant for EDSS low ( $<3.0$ ) or EDSS high ( $\geq 6.5$ ). Three stable, three progressive and four healthy controls were used in these studies. PBMCs were isolated via Ficoll and stored in liquid nitrogen until use.

### **Reprogramming and Characterization of Human iPSCs**

Cryopreserved PBMCs were thawed for culture and reprogrammed to iPSCs via transduction of episomal vectors containing Oct3/4, Sox2, Klf4 c-Myc, and Bcl-xl factors<sup>42-44</sup>. Generated iPSC lines were cultured and expanded in E8 medium with recombinant human vitronectin proteins<sup>44,45</sup>. The iPSCs were characterized by cytology, karyotype analysis, and flow cytometry analysis as previously described<sup>42,43</sup>.

Cell colonies after reprogramming displayed human iPS cell morphology and abundant expression of TRA-1-60, a commonly used marker of undifferentiated pluripotent human stem cells. Generated iPSC lines also exhibited normal karyotype by standard G-banding (300-500 bands) karyotyping method<sup>46–48</sup>.

### **Differentiation of iPSCs-derived OPCs and OLs**

Human iPSCs were differentiated to OPCs and OLs as previously described<sup>49</sup>, with minor modifications of cell density.

### **Cell Sorting**

Cells were sorted based on expression of either PDGFR $\alpha$  or O4, depending on the experiment and maturation state of the cells. For PDGFR $\alpha$  expression, cells were sorted after 56 to 60 days of culture. For O4 expression, cells were sorted at approximately day 75. Cells were dissociated to a single cell suspension with TrypLE, filtered, Fc blocked with True Stain FcX (1:20, Cat # 422302, BioLegend), then stained with either PDGFR $\alpha$ /CD140a (1:10 Cat # 323506, BioLegend) or O4 (1:50 Cat # 130-117-357, Milteny), and cell density was adjusted for sorting. Sorted cells were placed in culture to recover and then treated as described for each experimental technique.

## **Immunofluorescence (IF) characterization**

After sorting, cells were cultured until analysis and then were fixed with cold 4% paraformaldehyde for 20 minutes, permeabilized with 0.2% TX-100 in 1X PBS for 20 minutes, blocked with 3% NGS and 0.1% TX in 1X PBS for an hour. MBP primary antibody was used at 1:500 dilution in blocking buffer and incubated at 4<sup>0</sup>C overnight, followed by washing and incubation with secondary goat anti-mouse Alexa Fluor® 488 (1:500) for 2 hours in blocking buffer. Nuclear staining was conducted with 1µM DAPI, incubated for 20 minutes in PBS. All steps with the exception of the primary antibody were performed at room temperature.

## **Cytokine treatment**

Bulk OPC cultures were treated with recombinant human IFN $\gamma$  (PeproTech) at a dose of 10 ng/ml from day 36 to day 82-85 or from day 60 to day 82-85, with a change of medium every 48 hours.

## **Flow cytometry analysis**

Cells were prepared as described above, followed by staining with antibodies for cell surface markers, PDGFR $\alpha$ /CD140a (1:10, Cat #323506, BioLegend), O4 (1:50, Cat # 130-117-357, Miltenyi Biotec), and GLAST (ACSA-1) (1:10, Cat # 130-095-814, Miltenyi Biotec). Viability was determined by a Live/Dead Staining Kit (1:500, Cat #L34966, ThermoFisher).



For intracellular staining, cells were fixed and permeabilized with the Foxp3 transcription factor staining buffer kit (Cat # 00-5523-00, eBioscience) according to the manufacturer's protocol, followed by staining of the intracellular marker Olig2 (1:50, Cat # MABN50A4, EMD Millipore).

### **Co-culture of Neurons and OPCs**

Cultured cells were harvested at day 84 and were seeded in one of two compartmentalized chambers, which are separated by micro-channels as previously described<sup>50</sup> at density of 10,000 cells. The second chamber was seeded with neurons, generated from hESC (H9 cell line), and the axons from the neurons protrude through the micro-tunnels, providing a substrate for the myelinating OLs. Approximately 30 days later, cells were fixed with ice cold 4% paraformaldehyde for 20 minutes followed by washing with 1X PBS. Cells were then blocked with 3% normal goat serum and 0.1% Triton-X in PBS for 1hour, and stained with primary antibodies of mouse SMI99 MBP (1:50, Cat#SMI-99P, Covance), chicken NF (1:200, Cat# NF-M, Aves Lab Inc), and rabbit GFAP (1:1000, Cat#Z0334, Dako), overnight at 4°C. Samples were washed three times after incubation with primary antibody with 1X PBS, and then appropriate secondary antibodies conjugated with Alexa Fluor (488 mouse, 555 chicken, and 647 rabbit, all at 1:250, Invitrogen) were incubated for 1 hour and 30 minutes at room temperature.

Cells were incubated for 5 minutes with 1 $\mu$ M DAPI (4', 6-diamidino-2'-phenylindoldihydrochloride; Invitrogen) as a nuclear counter-stain.

Zeiss live-cell inverted microscope (Axio Observer; Zeiss, Germany) was used for imaging at 20X magnification.

### **Quantitative PCR**

RNA was isolated via an RNeasy kit (Qiagen) followed by cDNA preparation with an iScript cDNA Synthesis Kit (Bio-Rad). Reactions were conducted with iQ SYBR Green Super Mix (BIO-RAD) with primers manufactured by Integrated DNA Technologies (Coralville, IA, USA). Primer sequences were: Human GAPDH (Forward 5'-AAG GTG AAG GTC GGA GTC AAC-3', Reverse 5'- GGG GTC ATT GAT GGC AAC AAT A-3'), Human MBP (Forward 5'-CTA TAA ATC GGC TCA CAA GG-3', Reverse 5'-AGG CGG TTA TAT TAA GAA GC-3').

### **RNA sequencing**

OPC cultures were FACS-sorted for expression of PDGFR $\alpha$  as above, replated and cultured with or without the addition of 10 ng/ml of IFN $\gamma$  for 16 hours, followed by RNA isolation (RNeasy Plus mini kit, Qiagen). Libraries were prepared using the Illumina TruSeq Stranded Total RNA LT Sample Prep and were then sequenced on an Illumina HS2500 and Novaseq6000.

## RNA-Seq Analysis

Transcript counts were quantified by pseudo-alignment against the human transcriptome (GRCh38.p13) using Salmon v1.1.0<sup>51</sup>, after filtration of ribosomal RNA transcripts using BBSplit. Raw counts were imported into R v3.6.2 and aggregated to gene level counts with tximport v1.14.0<sup>52</sup> and then DESeq2 v1.26.0<sup>53</sup> was used to normalize, perform exploratory analysis, and test for differential gene expression. Volcano plots were generated with the Bioconductor package EnhancedVolcano v1.4.0 (<https://github.com/kevinblighe/EnhancedVolcano>). The functional analysis was generated through the use of Ingenuity Pathway Analysis (Qiagen) using thresholds of adjusted p value < 0.05 and Log<sub>2</sub> FC less than -1 or greater than 1<sup>54</sup>. The shell scripts and R script used to quantify and analyze are available upon request. Raw and processed data are publicly available: (GEO; <https://www.ncbi.nlm.nih.gov/gds>) with accession number GSE147315.

## Transmission Electron Microscopy (TEM)

Cells were fixed in 2.5% glutaraldehyde, 3mM MgCl<sub>2</sub> in 0.1 M sodium cacodylate buffer, pH 7.2 for one hour at room temperature. Samples were then rinsed and postfixed in 1% osmium tetroxide, 0.8% potassium ferrocyanide in 0.1 M sodium cacodylate for 1-2 hours on ice in the dark. The samples were then rinsed in 100 mM maleate buffer, followed by incubation with uranyl acetate (2%) in 100 mM maleate for 1 hr in the dark and then were dehydrated in a graded series of ethanol concentrations, and embedded in

Eponate 12 (Ted Pella), then polymerized at 37<sup>0</sup>C for 2-3 days followed by incubation at 60<sup>0</sup>C overnight. Thin sections, 60 to 90 nm, were cut with a diamond knife on the Reichert- Jung Ultracut E ultramicrotome and picked up with 2x1 mm formvar copper slot grids. Grids were stained with 2% uranyl acetate in 50% methanol followed by lead citrate and observed with a Philips CM120 TEM at 80 kV. Images were captured with an AMT CCD XR80 (8 megapixels camera - side mount AMT XR80 – high-resolution high-speed camera).

## **Statistical Analysis**

A statistical analysis was conducted on the percentages of cells expressing O4 or Olig2 between and among controls and MS patients (categorized as stable or progressive) with and without IFN $\gamma$  treatment (untreated, at day 36 and at day 60) using generalized estimating equations (GEE) to account for inclusion of multiple samples per patient. Statistical tests were two-sided, and significance was determined using a p-value less than 0.05. All analyses were performed using R (version 3.10; [www.R-project.org](http://www.R-project.org))

## 2.3 Results

### **iPSCs successfully differentiated to mature OLs**

PBMC-derived iPSCs were validated as described in methods and then cultured under a standard protocol to generate OL lineage cells. The progression of differentiation occurs over a lengthy time period of 80-90 days for full maturation (Fig 1). This process is shown in a sample phase contrast, which demonstrates the morphologic change associated with differentiation to oligo lineage cells in Fig 2. To more definitively validate the identity of the oligo lineage cells, we intensively characterized them using a variety of complementary methods. Throughout the culture period, we monitored cells for expression of canonical markers, transcription factors, and ultimately, the critical evidence of OL lineage, namely production of myelin and functional wrapping. Specifically, we characterized the phenotype of our cultures at distinct time points (OPCs, immature and mature OLs), to monitor development. Towards the end of cultures (approximately days 80-85) as cells are reaching maturity, we analyzed cells for the expression of characteristic markers.

As Olig2 is a critical transcription factor for the development of OLs, and MBP is the hallmark of a mature OL, we analyzed these in depth. As shown in Fig 3A, cells express both of these markers. OLs exhibit a characteristic morphology in their mature state, and Fig 3B shows highlighted OLs with both this morphology and a concomitant expression of MBP. These figures help to confirm the identity of the cells.

To validate the expression of O4, cultures were first sorted by FACS based on the expression of O4 and placed them in culture to mature fully. After culture, cells were then immunostained with O4. As shown in Figure 3C the cells retained expression through maturity, validating both the expression of the marker and the antibodies used for analysis. The critical function and hallmark of OLs is the production of myelin and wrapping of axons. We therefore assessed this process in different formats.

To assess myelination of axons, OL cultures were replated in a split chamber system, in which the OLs were plated in one chamber and neurons were cultured in a separate chamber, while their axons are allowed to migrate through microtunnels into the OL chamber. After approximately 30 days of co-culture, the chambers were subjected to immunofluorescence (IF) analysis with antibodies to MBP and neurofilament (NF). As shown in figure 3D, mature OLs were able to contact and apparently ensheath axons. To conduct an ultimate identification of wrapped axons, we employed electron microscopy to reveal the structure.

Figure 3E shows representative images of myelinated axons with compact lamellar myelin. Similar results were obtained from all donors. As a further confirmation of the identity of the cells, we conducted qPCR analysis for MBP expression, which provides a specific genetic confirmation of expression of this hallmark molecule. As shown in Figure 4, OLs had a high level of expression of MBP. Taken together, these data show that iPSCs from MS patients differentiated into OPCs, which then could be further differentiated to functionally mature OLs.

### **iPSCs from all patients differentiated into OLs**

Having validated the experimental process and identity of the cells, we next conducted a more high-throughput and quantitatively comparable analysis of the generation of OLs from iPSCs. To this end, we cultured and differentiated iPSCs from the six patients and four healthy controls, for approximately 85 days to a mature OL stage, and then profiled large populations by flow cytometry using O4 and Olig2 as identifying markers. O4 has been used to identify and characterize oligo lineage cells from tissue of healthy individuals and from chronic lesions of MS patients<sup>55,56</sup>. All MS iPSC lines expressed O4, albeit to different extents, which were similar to those reported previously<sup>32</sup>. The results of these analyses showed effective differentiation of all samples, with a broad range of efficiency of differentiation and maturation within (upon repeat culture) and across individuals. Figure 5 shows the

efficiency of differentiation for each donor.

Some cell lines demonstrated higher degrees of variability than others, but neither variability nor final efficiency of maturation was statistically linked with one cohort (Figure 5A). In order to compare among groups, we analyzed the averages of each group with donors of each cohort combined (Figure 5B). While there was a trend to lower efficiency in the progressive group compared to healthy controls, there was no significant difference in the aggregates. We similarly analyzed the comparison of Olig2 expression, with similar results (Figure 5C), in which a trend towards a decrease in progressive samples was present, but it did not reach significance. Taken together, these results demonstrated that iPSCs from all donors could ultimately differentiate into mature OLs.

### **Chronic exposure to low-dose IFN $\gamma$ inhibits OPC differentiation**

An important consideration in myelin repair in a chronic disease is that the CNS may not be in a homeostatic state in MS, and that either overt or smoldering inflammation may be present. To investigate the effects of an inflammatory environment on the differentiation of iPSCs into OPCs, we next assessed the process in the presence of sustained exposure to low-dose IFN $\gamma$ . Parallel cultures of untreated and treated cells were established, with the IFN $\gamma$  provided during the critical period of development, spanning from



day 36 to final analysis , or for some experiments from day 60 to final analysis. At the end of culture, cells were analyzed by flow cytometry for differentiation markers as established in figures 4 and 5.

Figure 6 shows the results of differentiation of each individual MS patient, with and without IFN $\gamma$ . Figure 7A shows a combined analysis of progressive, stable and healthy control comparisons, and figure 7B shows the effects of IFN $\gamma$  treatment on the differentiation of the donors, separated by groups, with replicates for each donor averaged. As shown in the graphs, IFN $\gamma$  treatment initiated early during culture profoundly inhibited the development of O4+ OLs in all groups. While iPSCs from some patients differentiated more efficiently than others, the inhibition was uniformly robust, regardless of the nature of disease in the donor. The culture requires a lengthy process, and as cells transition through phases of progenitor plasticity during the course of differentiation, we sought to determine whether IFN $\gamma$  exposure might produce different effects on cells that were not treated until they had reached the early OPC stage. Interestingly, the inhibitory effects were significantly milder when treatment was delayed to day 60, highlighting a critical period of vulnerability during the early stages (Figures 6-7). A similar effect was observed on the expression of Olig2, a transcription factor necessary for activation of the genetic program of OLs (Fig 8), again supporting the inhibitory effect of IFN $\gamma$  on the development of mature OLs.

As a genetic confirmation of the impact of IFN $\gamma$  on terminal differentiation, an analysis of MBP expression demonstrated that while untreated cells had high levels of MBP, consistent with mature OL status, those that were exposed to IFN $\gamma$  had minimal detectable levels, furthering the evidence that OL development was inhibited (Figure 9).

In sum, these data, along with the data from Figures 4-5, show that iPSCs from all groups effectively mature to OLs, but exposure to IFN $\gamma$  significantly derails this process.

### **Sustained exposure to low dose IFN $\gamma$ does not restrain cells at OPC stage but rather redirects lineage phenotype**

Consistent with previous studies, we observed no overall increase in cell death in treated cultures, even when cells were exposed to low-dose IFN $\gamma$  during the high vulnerability period from day 36 to 60 (Figure 10). Thus, in order to account for the decrease in OLs, we evaluated other populations to identify one with a concomitant increase. Previous rodent studies showed that IFN $\gamma$  treatment of progenitors led to an increase in PDGFR $\alpha$  progenitors and a decrease in mature OLs by overriding cell cycle exit<sup>39</sup>. We thus quantified PDGFR $\alpha$  expression at the end of the cultures, but did not find a universally similar result in the human cells, regardless of whether treatment started at d36 or d60.

As expected at this timepoint, PDGFR $\alpha$  expression was low in untreated cultures, as OPCs have matured into OLs and thus lost expression by this phase. Treatment with IFN $\gamma$  did not increase the fraction of cells expressing PDGFR $\alpha$ , with the exception of the progressive samples, which while reaching a low level of significance, the percentage of all remained very low (<5%) and thus could not compensate for the loss of the large OL population (Figure 11).

As astrocytes commonly arise from mixed cultures, another possibility was that IFN $\gamma$  treatment skewed early cells towards an astrocytic profile. To quantify percentages of astrocytes we performed flow cytometry analysis using the GLAST (ACSA-1) marker. Our results showed IFN $\gamma$  exposure significantly reduced this population as well (Figure 12).

As development of OL and astrocyte lineages was suppressed, we next investigated the development of neuronal lineages in the presence of IFN $\gamma$ , via expression of  $\beta$ III tubulin (TUJ1). In sharp contrast to the suppressive effects on astrocyte and oligo lineages (Figures 12-13A), IFN $\gamma$  exposure led to a concomitant, significant increase in the percentages of cells expressing TUJ1, with similar patterns linked to time of exposure (i.e., from day 36 or day 60) (Figure 13B).

## **Short-term IFN $\gamma$ treatment upregulates immune response pathways.**

To better understand the effects of IFN $\gamma$  treatment on OPC differentiation, we sought to monitor early transcriptional changes through RNA sequencing. To this end, PDGFR $\alpha$ <sup>+</sup> cells were sorted at the OPC stage (day 60), allow to recover in culture, and then treated for 16-hour with low-dose IFN $\gamma$ . We found a pronounced effect on the transcriptome of OPCs with over 3200 differentially expressed genes; 1749 were upregulated and 1453 downregulated (Figure 14A-B). The most induced and highly significant genes were largely those traditionally associated with an inflammatory response such as TAP1, CXCL9, and CD74 (Figure 13B, Table 1). Among the highly upregulated genes, we found early interferon response genes: STAT1, IRF1, IRF2, IRF7, and IRF9, peptide processing genes: TAP1, TAP2, and ERAP1, immunoproteasome component genes: PSME1, PSME2, PSMB8, and PSMB9, antigen presentation genes class I: HLA-A and HLA-B, and class II: CIITA, (Figure 14C, Table 1).

Consistent with these findings, functional analysis of differentially regulated genes showed most significant enrichment for antigen presentation and immune activation (Fig 14D). In their rodent studies, Chew et al., 2005 showed that IFN $\gamma$  kept OPCs in the progenitor state by overriding the cell-cycle exit, which they demonstrated by showing upregulation of cell-cycle proteins on IFN $\gamma$ -treated cells <sup>39</sup>.

Our results did not show a similar differential expression of cell-cycle genes (Figure 14C), indicating that a different mechanism may be operational at this timepoint in human cells. Consistent with suppression of OL development, however, upregulation of DAAM2, which was recently shown to play a role in suppressing OPC differentiation but has not previously been linked with IFN $\gamma$  signaling in OPCs <sup>57</sup>, was observed ( $\text{Log}_2\text{FC} = 1.97$ , adjusted p value =  $3.73\text{E-}07$ ). Thus, treatment of progenitors at the PDGFR $\alpha$ + OPC state led to an early, robust upregulation of immune and inflammatory response genes

## 2.4 Discussion

The results of our studies revealed a number of novel and intriguing findings that may help to advance the development of strategies for neuroprotection and/or repair. Because of the inaccessibility of cells of interest in MS and the long course of disease development, creating platforms to study human cells derived from different patients will be critical to these advances. In order to generate a relatively high throughput system that allows intensive investigation of these cells, culture systems have been established, and initial results have reported the feasibility of differentiation into functional OLs from iPSCs.

Both intermediate OPCs and mature OLs were generated from people with MS as well as from healthy controls. Importantly, in addition to the demonstration of an OL phenotype, the cells were shown to successfully myelinate a shiverer mouse, an animal model that cannot form compact myelin due to MBP deficiency<sup>20</sup>. Consistent with these reports, our data also showed that iPSC from people with either stable/low disability or progressive/high disability MS differentiated into OPCs and OLs, expressing canonical markers that span the timeline of development and ultimately producing MBP and myelin that wrapped axons.

OPCs have been identified at the site of MS lesions, and the apparent ability of progenitors to mature contributes to the great remaining mystery of why the process of remyelination fails, and as a corollary why treatments to promote remyelination have not achieved greater success in human trials<sup>58</sup> despite promising animal modeling<sup>33,59</sup>.

One critical factor that is often not considered in the drug screening process or in vivo models is the diseased environment. The local milieu includes secreted cytokines by invading cells as well as by resident diseased cells that affect balance of anti- and pro- inflammatory signals, growth factors and debris clearance<sup>60,61</sup>. For example, recent studies have shown that iPSC-derived neural progenitor cells (NPC), from people with primary progressive MS (PPMS) did not provide adequate trophic or protective support for rat OPCs in culture<sup>62</sup>. Additional work from the same group later showed that diseased NPCs highly upregulated senescence genes, with HMGB-1 as a potential culprit in the failure of diseased NPCs to support rat OPC growth, from both local and migrating cells<sup>63</sup>.

In addition to the absence of trophic effects, the disease environment is also challenged by the presence of inflammation, which is a prominent feature in early-stage MS. An array of proinflammatory cytokines have been implicated, including IL-17, IL-22, IL-1, IL-12, tumor necrosis factor- $\alpha$ , and IFN $\gamma$ <sup>64</sup>. IFN $\gamma$

is increasingly recognized as a driving factor in the pathology for its ability to amplify an inflammatory response by orchestrating the upregulation of CCL5, MIF, and CCL27 cytokines via activation of astrocytes and T-cells <sup>65</sup>. The effects also prevent OPC differentiation and remyelination, in part because IFN $\gamma$  foment a hostile environment that is not conducive to regeneration by inducing cell death (at high concentrations) i.e. STAT1/IRF-1 pathway <sup>36</sup>, inhibiting cell cycle exit <sup>39</sup>, and inflicting endoplasmic reticulum stress <sup>41</sup>.

To mimic an inflammatory disease-type environment, we sought to investigate effects of chronic exposure of low-dose IFN $\gamma$  on our cultures. In a long-term culture setting, a very low dose was sufficient to dramatically inhibit the maturation of OLs, and the earlier the phase of culture and the longer the cells were exposed, the greater the effect. Our studies are consistent with previous findings in that IFN $\gamma$  has a milder effect on immature rodent OLs, and a minimal effect on mature OLs <sup>38,66</sup>. The mechanisms behind this difference remains unclear, but highlights a critical vulnerable stage of development. Consistent with previous studies demonstrating that low-dose IFN $\gamma$  did not promote apoptosis <sup>39</sup>, our data also indicated no difference in cell death between untreated and interferon groups, which suggests that at least in low levels of inflammation, killing of the OPCs may also not be the primary cause of failure to remyelinate. It is interesting to speculate that the profound inhibitory effect that IFN $\gamma$  exerts on OPC differentiation could contribute to the failure to remyelinate in a disease



setting. The presence of OPCs at the sites of injury <sup>15,21</sup> and evidence of early remyelination in MS <sup>16,67</sup> strongly suggest that the capability to replace OLs and repair myelin is present. One possibility thus is that OPCs do migrate properly to site of injury to begin the repair process, but ultimately are halted in the differentiation process by the presence of a pro-inflammatory environment, and the effects of even low-dose cytokine could be sufficient to effectively shut down the repair process, in theory.

IFN $\gamma$  is a potent and pleiotropic cytokine that has been shown to affect mitochondrial function, which is considered a cause of axonal degeneration that accompanies failed remyelination <sup>68,69</sup>. Interferon also induces oxidative stress, which in turn can cause DNA damage <sup>1</sup>. A significant implication of these data is that screening strategies to discover agents to promote endogenous differentiation may benefit from considering their effects under chronic low-grade inflammation, since therapies that promote OPC differentiation and/or survival in a homeostatic state may not be as effective in the presence of inflammatory molecules, and testing in the presence of such an environment may help to identify more potent and effective agents. It is becoming clearer that progenitors of most cell types are not unalterably committed to a linear and unwavering path but rather may be influenced by environmental conditions, including growth factors and cytokines. In support of this notion, for example, previous work showed that IFN $\gamma$  inhibited differentiation of rodent A2B5+ progenitor cells, reducing the number of

mature O1+ oligodendrocytes, while enhancing proliferation of immature OPCs<sup>39</sup>. Our data is consistent with the suppression of maturation, but in our long-term culture of human cells, there was neither retention nor selective proliferation of early progenitors overall, but rather, the acquisition of a neuronal phenotype in at least a portion of cells. This finding parallels a previous discovery in microglia, in which exposure to a low-dose of IFN $\gamma$  led to the acquisition of a neuronal-like phenotype<sup>70</sup>. Consistent with these findings, another report showed *in vitro* that IFN $\gamma$  induced neuronal differentiation of neural stem cells in a dose-dependent manner. Together, these data support the notion that IFN $\gamma$  inhibits the development of oligo lineage cells while promoting the differentiation into a neuronal lineage<sup>71</sup>.

This prevalent plasticity in various cell types in response to the environment highlights a critical point in the ability of cells to adapt to changing circumstances and local influences and possibly serve different roles under conditions of disease or infection, for example. One hypothetical consequence of such plasticity is that as cells convert to different phenotypes, the availability of functional cells needed to repair could be depleted. If the OPCs and progenitors follow a different lineage pathway, then they would be unavailable to become OLs in this setting, for example. Numerous lines of evidence indicate the ability of progenitor cells to acquire different characteristics, the mechanisms behind the redirection are less clear.

As a means to begin to gain a greater understanding of the transcriptional basis for reprogramming in people, we undertook a transcriptomics analysis to identify genetic patterns of expression in a homeostatic and IFN $\gamma$ -exposed states. The baseline genetic profile confirmed the identity of the PDGFR $\alpha$  sorted cells as OPCs. Interestingly, IFN $\gamma$  exposure led to the differential expression of a number of genes in diverse pathways. Consistent with our observed effects on decreased myelin production, our screen identified an upregulation of one gene of interest, DAAM2 (Dishevelled Associated Activator of Morphogenesis 2), a Wnt signaling downstream effector. DAAM2 suppressed remyelination after white matter injury in rodents and humans<sup>57</sup>, suggesting that this pathway may be a possible candidate for genes that could theoretically contribute to the observed effect.

Another study demonstrated a correlation between decreased DAAM2 expression and improvements in remyelination in Guillain- Barre syndrome<sup>72</sup>, suggesting that IFN $\gamma$  may activate specific pathways redirecting the process. DAAM2 is also thought to be important for dorsal patterning in the development of the spinal cord<sup>73</sup>, which is also consistent with these findings. Further investigation into the significance of this pathway in this setting would be necessary to parse out its function, however, but it is interesting to speculate that activation of specific programs of gene expression by cytokine exposure could dramatically alter the cells. An intriguing re-direction of OPC

function into an immune/inflammatory realm has been recently identified as well. Specifically, rodent OPCs exposed to IFN $\gamma$  acquired properties typically associated with the immune system. Termed “inflammatory OPCs” these IFN $\gamma$ –exposed cells upregulated molecules such as MHCs and further took on antigen presenting cell characteristics<sup>74,75</sup>. The implications of this process are profound, in that it suggests the possibility that OPCs at the site of a lesion could convert to an immune-like cell that stimulates cytotoxic T cells and perpetuates a damaging immune response, while similarly converting away from a cell type designed to repair damage. Our transcriptomics analysis demonstrated this conversion on at least the RNA level in all of the samples we tested. Our present data showed upregulation of interferon response genes such as STAT1, IRF1, IRF2, IRF7, and IRF9, all of which could contribute to the perpetuation of an inflammatory phenotype.

Aligning with the possibility of acquisition of antigen presenting cell characteristics, IFN $\gamma$  also led to upregulation of peptide processing genes of importance to the immune system, including TAP1, TAP2, and ERAP1, along with the immunoproteasome genes PSME1, PSME2, PSMB8, and PSMB9. Further, MHC molecules necessary for antigen presentation including class I HLA-A and HLA-B, and class II CIITA were all highly upregulated in response to IFN $\gamma$ . These results have intriguing implications in a disease setting in that it could be a means by which OPCs inadvertently worsen disease at the site at which repair is needed.

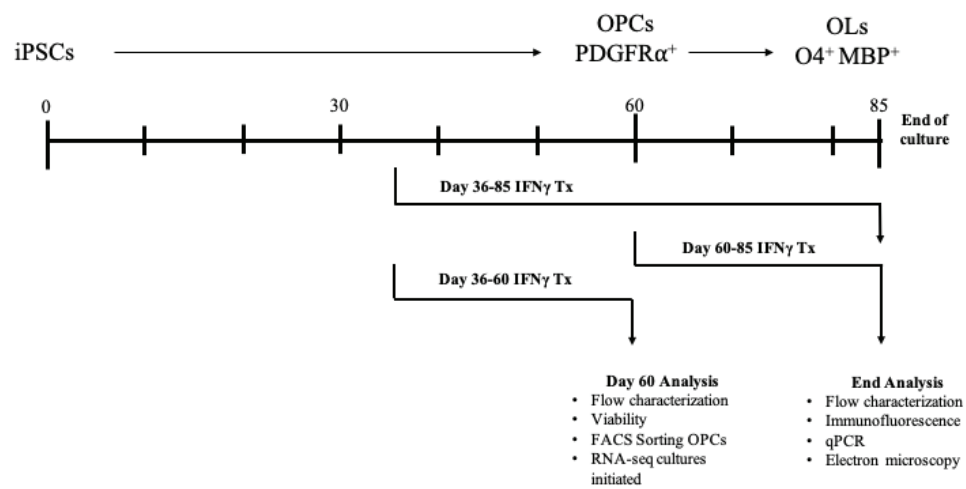
Gaining a greater understanding of OPC and OL function in people with MS is of paramount importance to uncovering mechanisms of disease and developing new targeted therapeutics. Taken together, our data demonstrate that in a nurturing environment, iPSCs derived from people with stable and progressive MS are capable of differentiating into mature OLs, but that this process is profoundly inhibited by chronic exposure to low-dose IFN $\gamma$ . Our data have significant clinical relevance as they suggest that the environment in which differentiation therapies are administered may profoundly affect the efficacy of the treatment, and ultimately, a combination approach of decreasing inflammation in the CNS may be necessary for OPC differentiating/remyelinating agents to reach their full efficacy.

**Table 1. RNA-seq analysis of gene enrichment of IFN $\gamma$  exposure in PDGFR $\alpha$  sorted OPCs.**

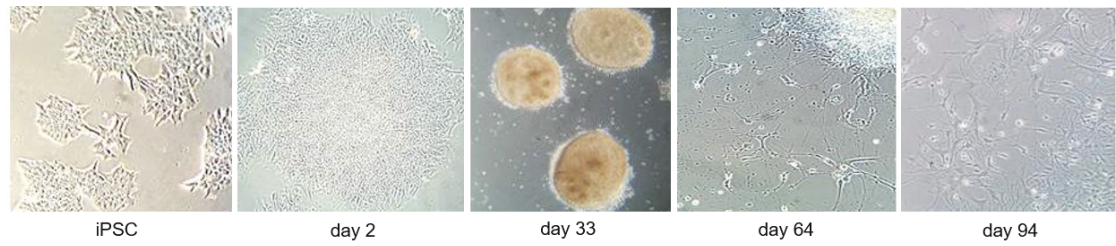
Gene	Log <sub>2</sub> FC	p-adj
IDO1	14.83555	8.17E-53
CXCL9	13.76216	1.18E-47
APOL1	11.90543	2.27E-32
GBP4	11.72463	1.44E-56
CXCL11	11.47197	1.15E-48
LGALS17A	10.60319	1.66E-20
XAF1	10.42001	5.05E-20
OAS2	10.10588	7.54E-75
CXCL10	10.05021	7.80E-116
GBP1P1	9.55261	1.86E-56
BATF2	9.54757	5.92E-52
GBP1	9.28494	3.37E-70
PLA2G2D	8.77572	3.87E-04
GBP6	8.58085	6.02E-07
CIITA	8.44938	1.06E-58
CEACAM1	8.42068	8.08E-19
TMEM140	8.39200	2.36E-28
USP30-AS1	8.33499	1.11E-18
ACSL5	8.33042	8.93E-12
GBP5	8.26802	4.57E-80

**Table 1** shows the 20 most upregulated genes detected by RNA-Seq after exposure to IFN $\gamma$ . Differentially expressed genes were sorted by fold change (Log<sub>2</sub>Fold Change). Adjusted *p* value is shown in the third column.

# Figures

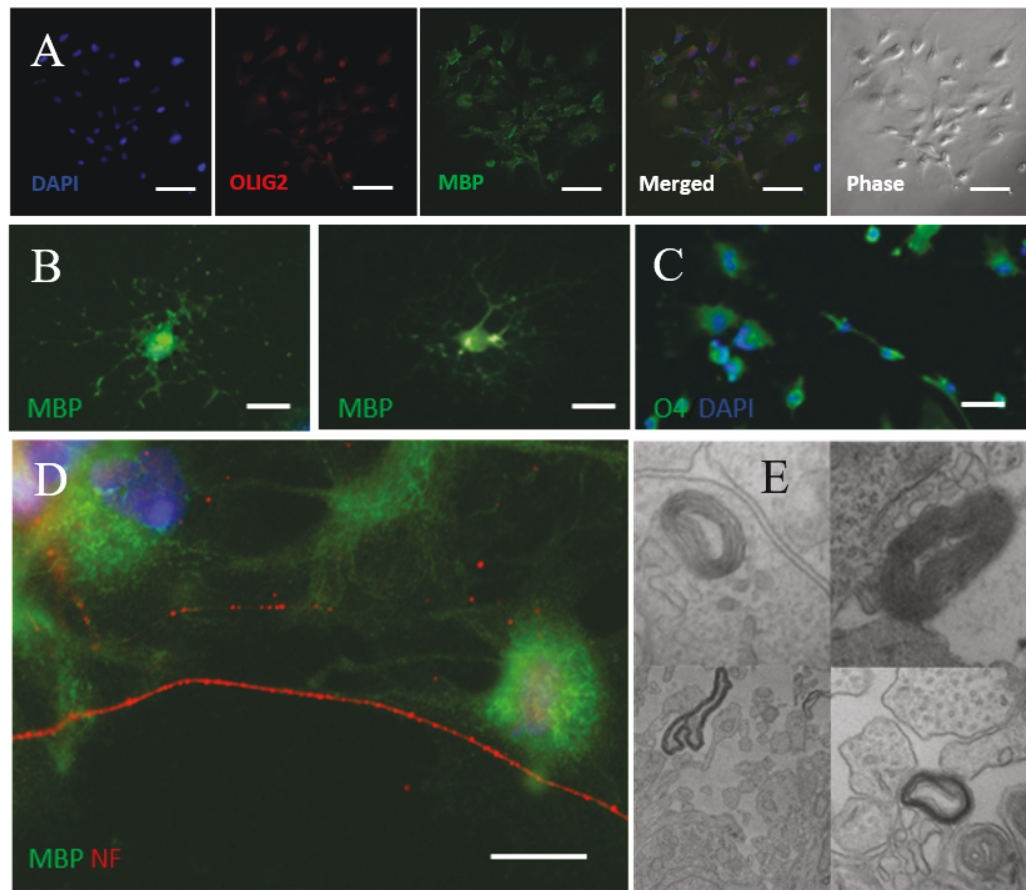


**Figure 1.** Schematic of experimental timeline



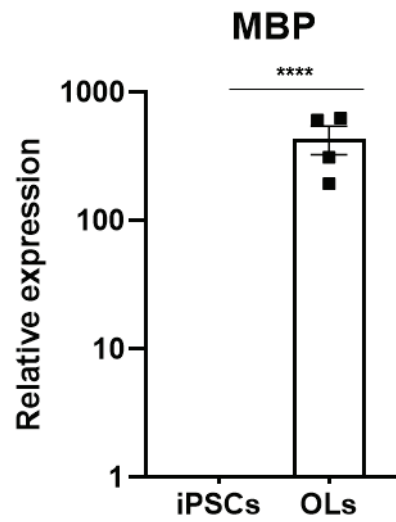
**Figure 2.** Phase contrast analysis demonstrating the morphological changes during the progression of OL development. 20X magnification





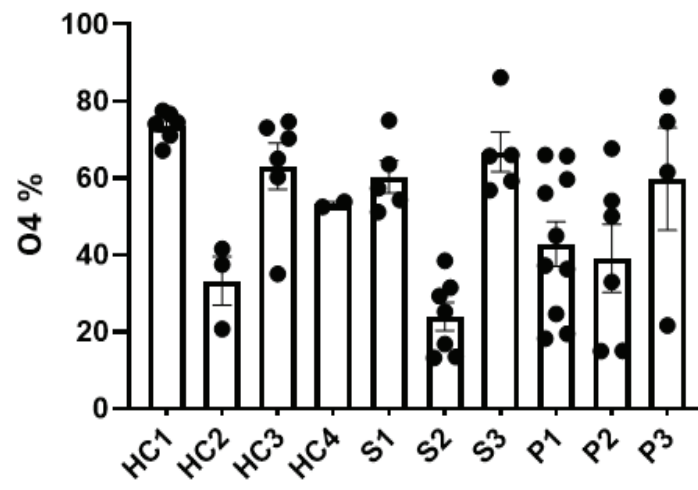
**Figure 3.** Validation of OL lineage identity was conducted during the course of the differentiation via multiple techniques. For analysis of purified populations, cells were FACS-sorted at two different time points. For **Figs 3A and B**, cells were FACS-sorted for PDGFR $\alpha$  expression at the OPC stage after 60 days of differentiation and analyzed after day 85 by immunofluorescence for expression of canonical markers. Cells were identified with the nuclear DAPI stain and labeled with antibodies for Olig2 and MBP as shown, with a merge in **Fig 3A**. A selected image showing myelin basic protein (MBP) staining with characteristic OL morphology is

highlighted in **Fig 3B**. **Fig 3C** shows the immunostaining of cells that were FACS-sorted for the expression of O4 at day 75 of culture, then re-cultured for an additional 9 days prior to analysis. As shown, O4 expression remained and was confirmed at day 84 of differentiation. To demonstrate functional myelination, two different approaches were undertaken. First, OLs at day 84 of differentiation from mixed cultures were co-cultured in a chamber format in which neurons remain on one side, but axons project through micro-tunnels to the other side where OLs are cultured. After three weeks of co-culture, cells were immunostained for MBP in green, and neurofilament in red. **Fig 3D** shows OLs contacting axons, with the appearance of wrapping. The second approach to validate the functional wrapping was transmission electron microscopy (TEM) analysis on OLs from mixed cultures as shown in **Fig 3E**. All images are representative samples encompassing all cell lines. Images 3A-E were taken at a magnification of 20X (scale bar = 50  $\mu$ m).

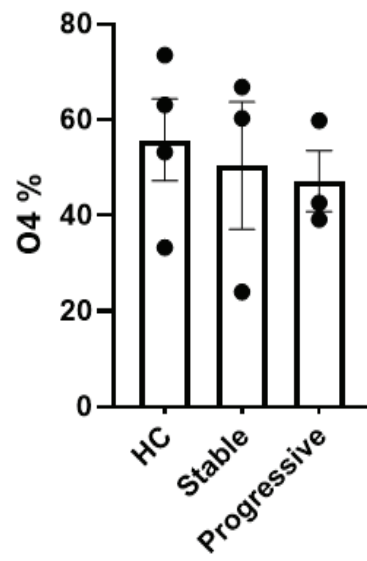


**Figure 4.** qPCR analysis shows high levels of expression of MBP compared to iPSCs as shown. Four cell lines in duplicate, normalized to GAPDH.

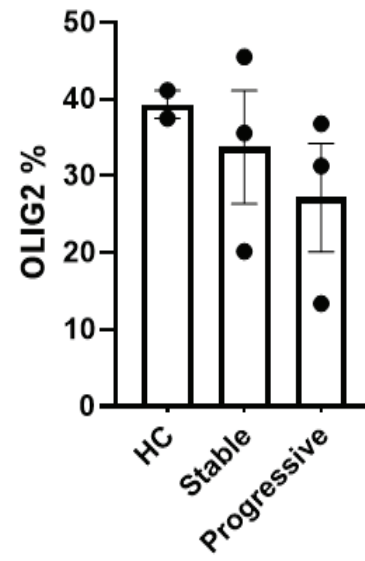
A



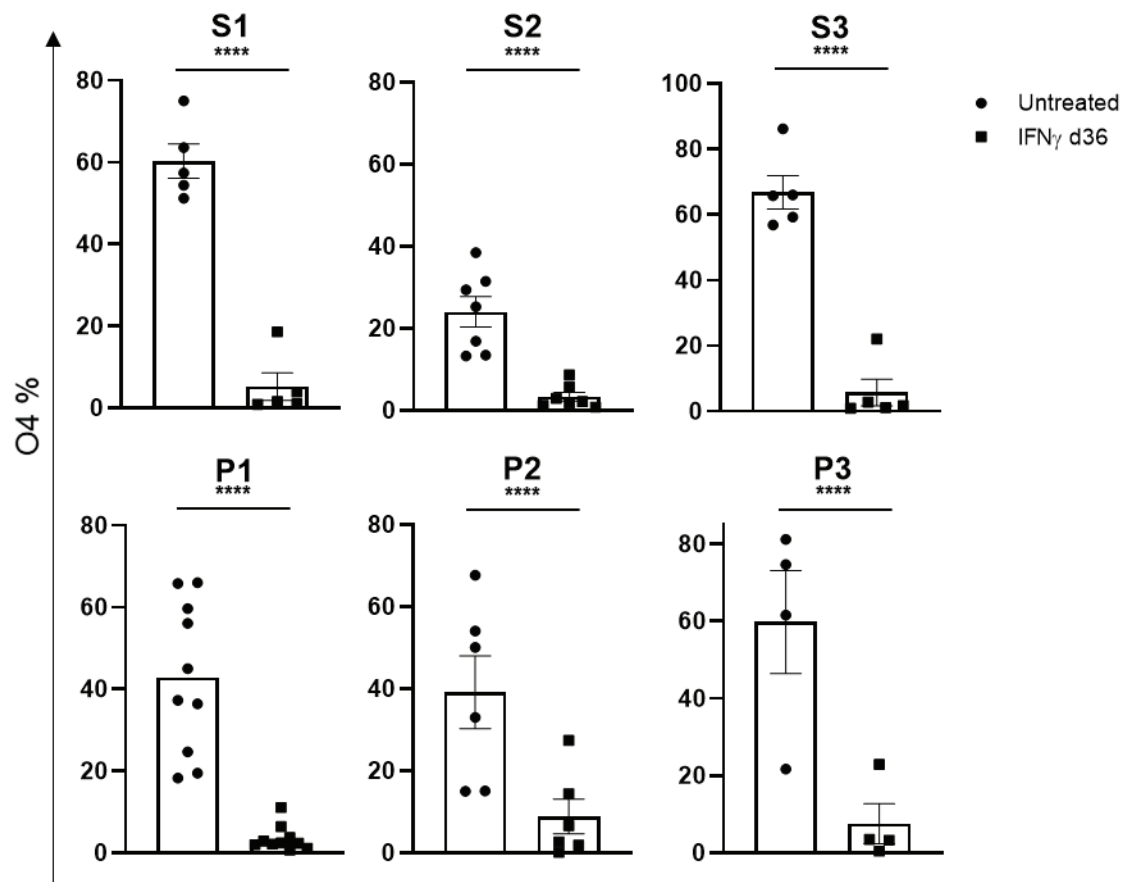
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C

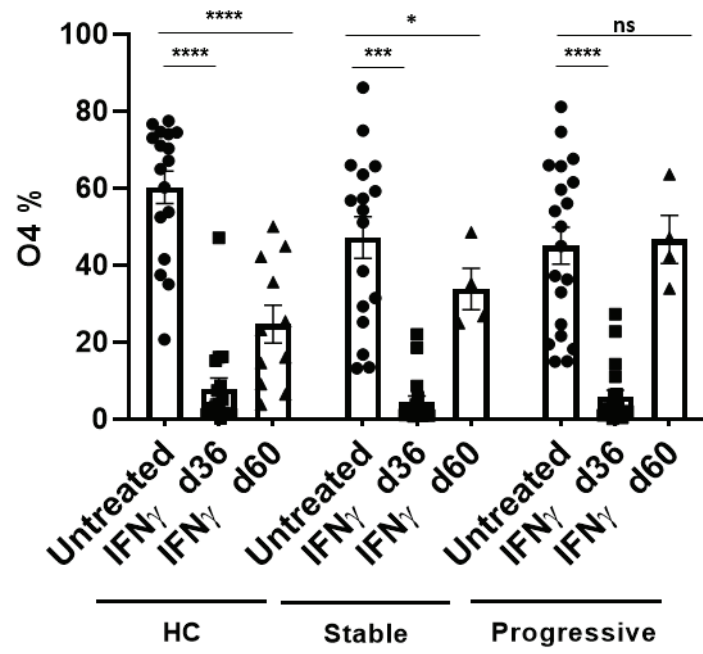


**Figure 5.** iPSC lines from all donors differentiate to OLs. To quantify and compare the differentiation of iPSCs into OL lineage cells from multiple donors in a more high-throughput manner, flow cytometry analysis was employed. Based on the validation in figure 2, O4+ and OLIG2+ expression was determined in cells from mixed cultures at approximately day 85 of differentiation. **5A** shows the percentage of O4 expression for each of the four healthy controls (HC1-4), and MS donors classified as stable (S1-3), and progressive (P1-3). Each dot representing one experiment per donor. Number of experiments per donor: HC1=6, HC2=3, HC3=6, HC4=2; S1=5, S2=7, S3=5; P1=10, P2=6, P3=4. **5B** shows the compilation of the data in Fig 5A, with the samples from each group combined and averaged. As in Fig 5A, the data are comprised of four healthy controls, three stable, and three progressive donors. Each dot represents the average for one donor. **5C** the compilation of the data of expression of Olig2 per group, with each dot representing one patient (not all samples were analyzed for Olig2). Number of donors per group: HC= 2, S=3; P=3, with 3-6 experiments per group. Significance was determined with generalized estimating equations with R, and using a p-value less than 0.05.

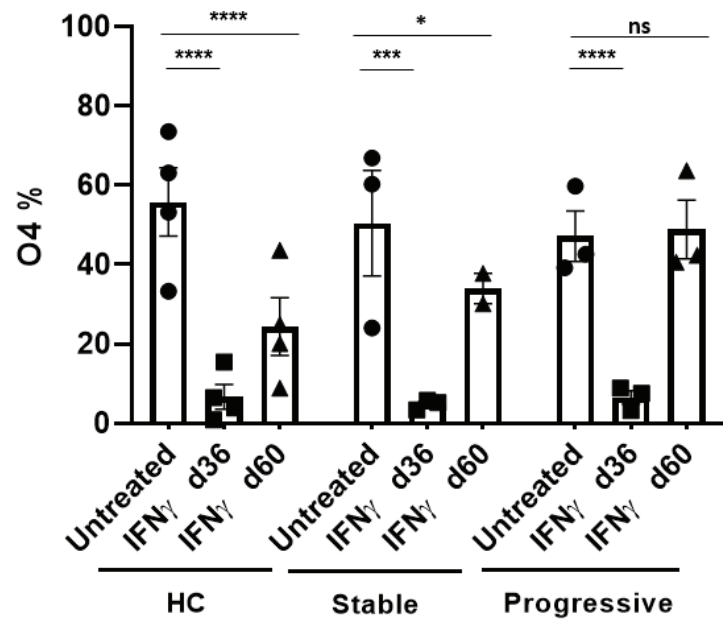


**Figure 6.** Sustained exposure to low-dose IFN $\gamma$  inhibits OPC differentiation in MS and healthy control groups. To assess the effects of low dose (10 ng/mL) IFN $\gamma$  on OPCs differentiation, mixed cultures were left untreated or treated from either day 36 or 60 to the end of culture, at approximately day 85. Flow cytometry analysis was then conducted to evaluate differentiation. Figure 6 shows the percentage of O4 expression in untreated and IFN $\gamma$ -treated cultures in lines from six individual MS donors, each with three to nine experimental replicas.

A



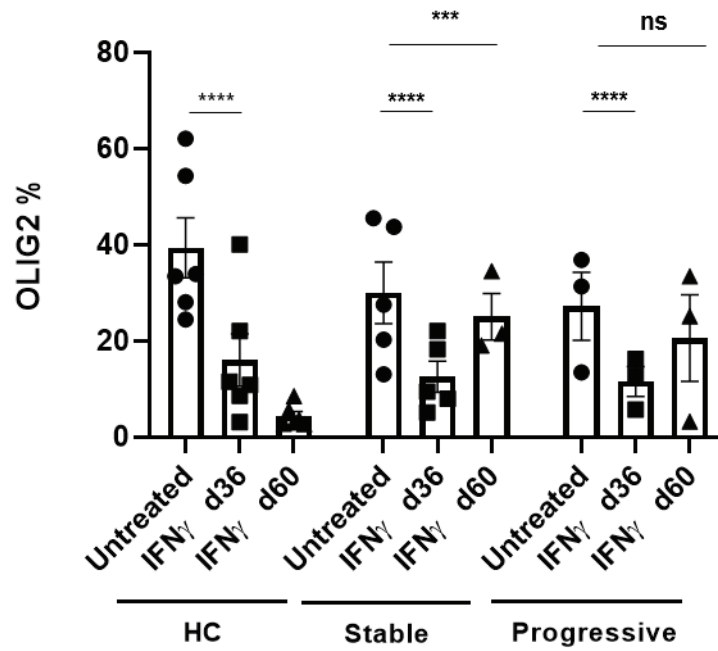
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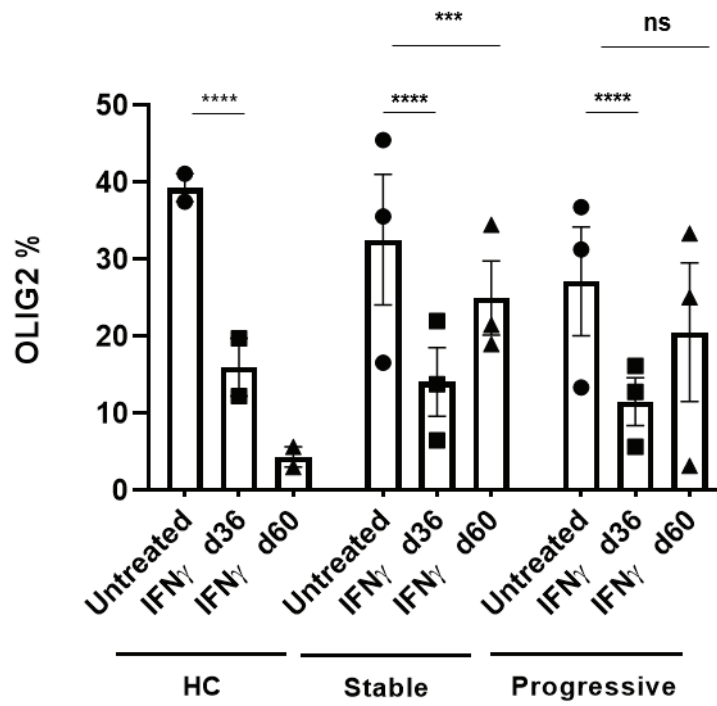
**Figure 7A.** Combined expression of O4 from both MS groups, alongside the combined HC group, all treated at either day 36 or 60 as noted. The control group consists of four donors with 11 to 14 experimental replicas, the stable group consists of three donors with 3 to 14 experimental replicas, the progressive group contains three donors with 3 to 17 experimental replicas, and the. **Figure 7B** shows the average combined expression of O4 per donor in each group



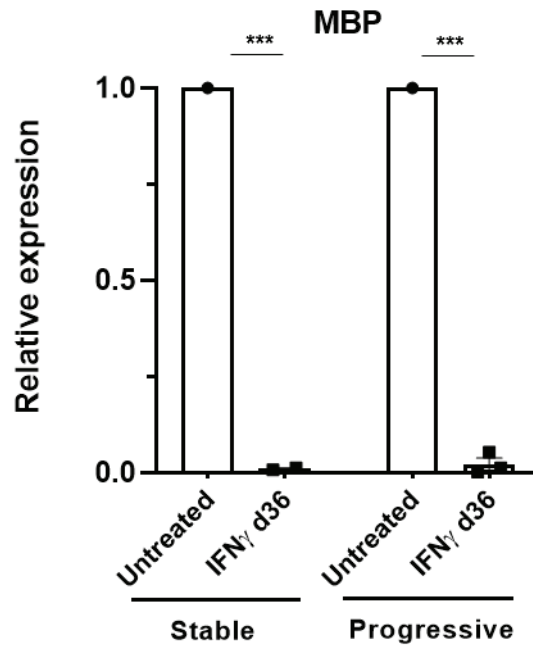
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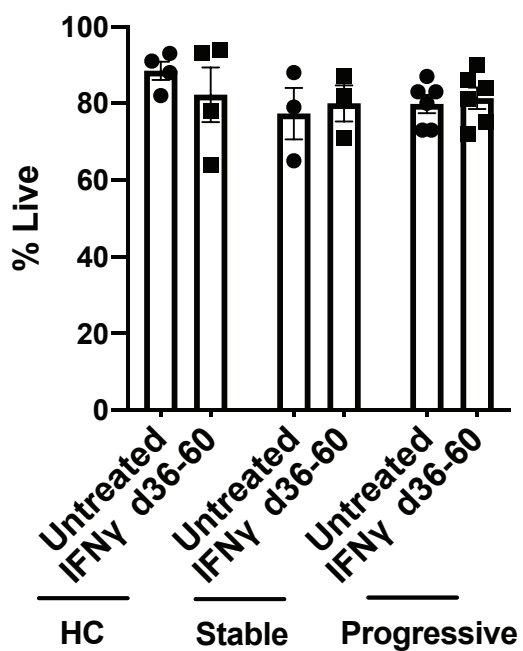
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**Figure 8A.** Combined expression of O4 from both MS groups, alongside the combined HC group, all treated at either day 36 or 60 as noted. The control group consists of two donors, each with two experimental replicas. The MS stable group consists of three donors, two of which have one replica. The progressive group consists of three donors. **Figure 8B** shows the average combined expression of OLIG2 per donor in each group. Significance was determined with generalized estimating equations with R, and using a p-value less than 0.05.

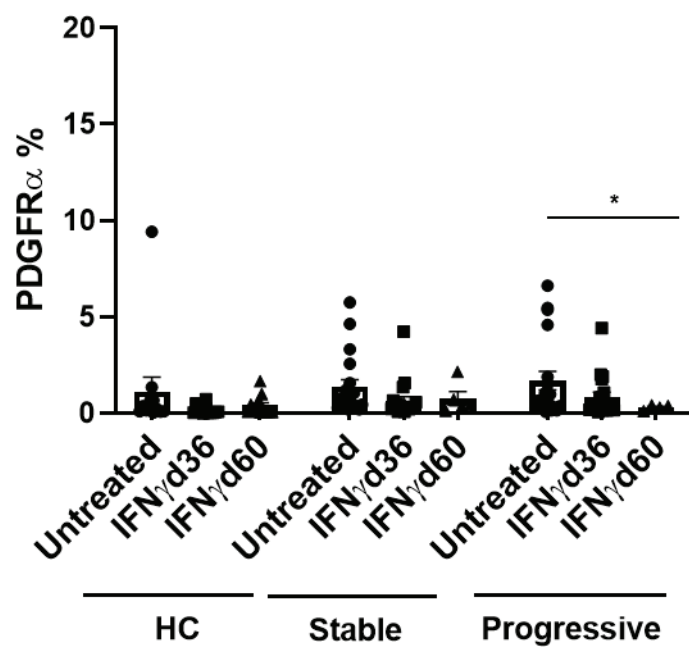


**Figure 9.** Further confirmation of the inhibition of OL maturation by the decreased expression of MBP in both MS groups (two donors for stable, three donors for progressive) as quantified by qPCR.

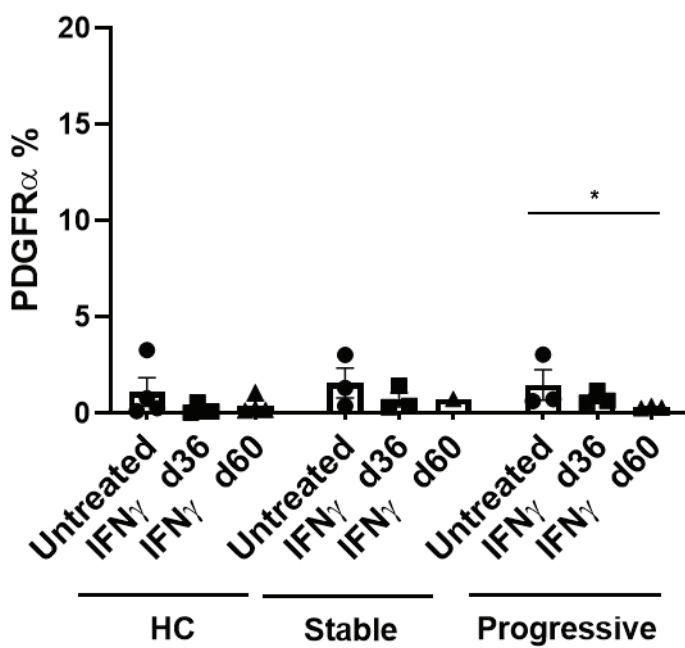


**Figure 10.** Percentage of live cells as determined by flow cytometry viability analysis, of untreated cells and cells treated with IFN $\gamma$  from day 36 to 60, which is a period of high vulnerability for the cells. Shown are the compiled and averaged results for each group, with each dot representing a single donor, with 1-2 donors per group, repeated 1-4 times.

A

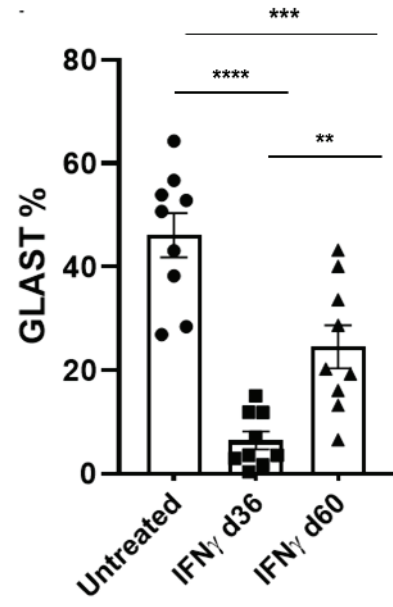


B

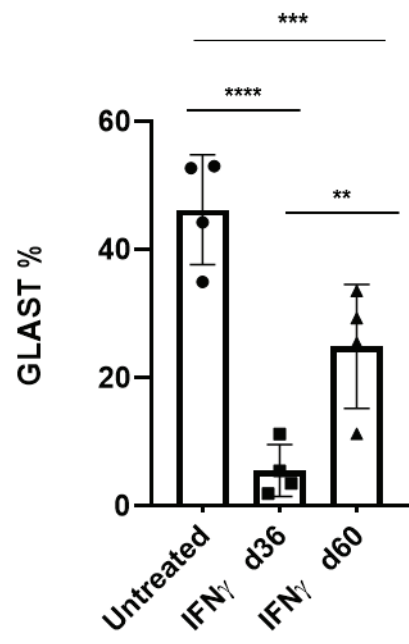


**Figure 11. Sustained exposure to low dose IFN $\gamma$  does not enrich for cells expressing PDGFR $\alpha$ .** After approximately 85 days of culture, the identity of the IFN $\gamma$ -treated cells was determined by flow cytometry. **11A** shows the late-stage culture expression of PDGFR $\alpha$  for each group in untreated and IFN $\gamma$ -treated cells, beginning at either day 36 or 60, as labeled. The healthy control group consist of 4 donors, each with 2 replicas; the stable group consists of three donors, each with five to eight replicas; the progressive group includes three donors with four to seven replicas of each. **11B** shows the average combined expression of PDGFR $\alpha$  per donor in each group.

A

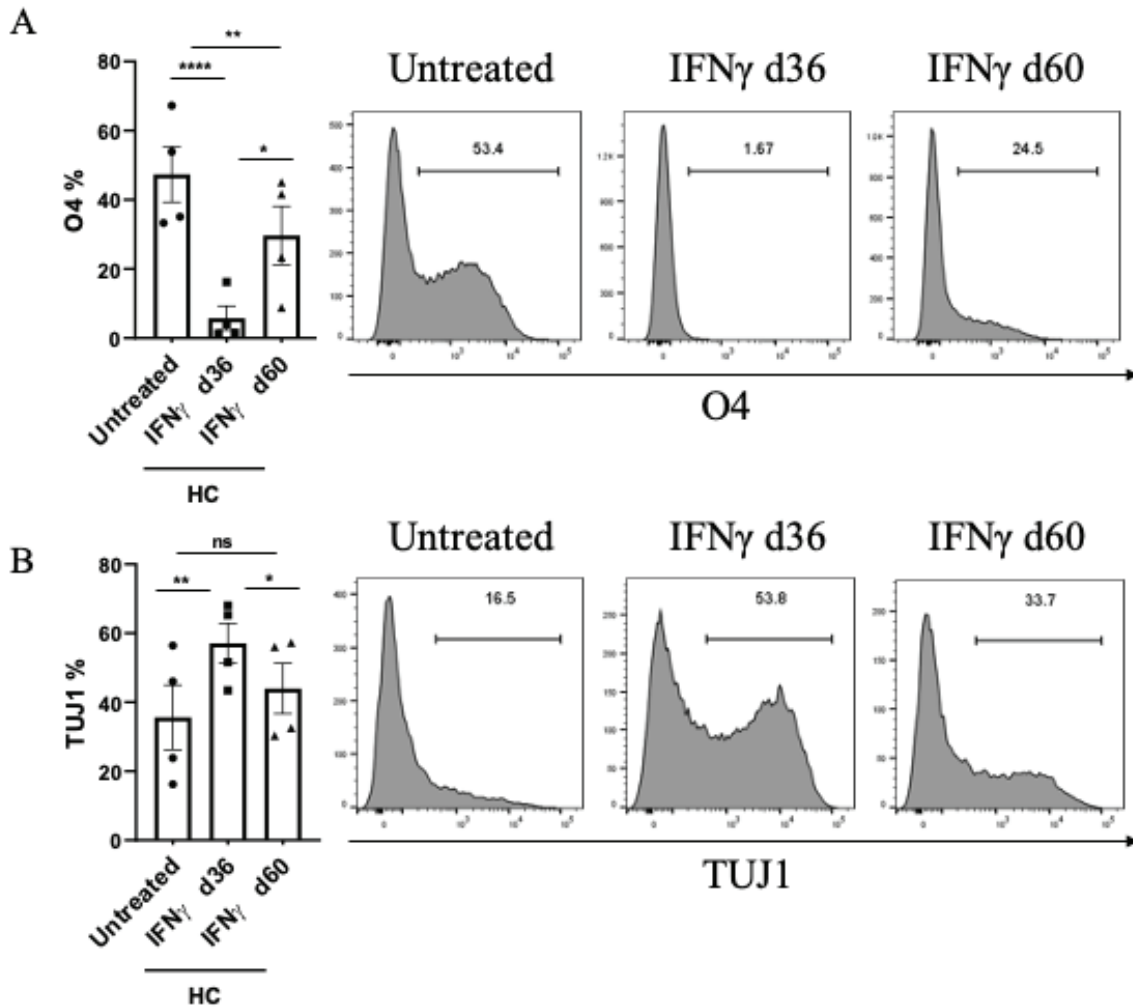


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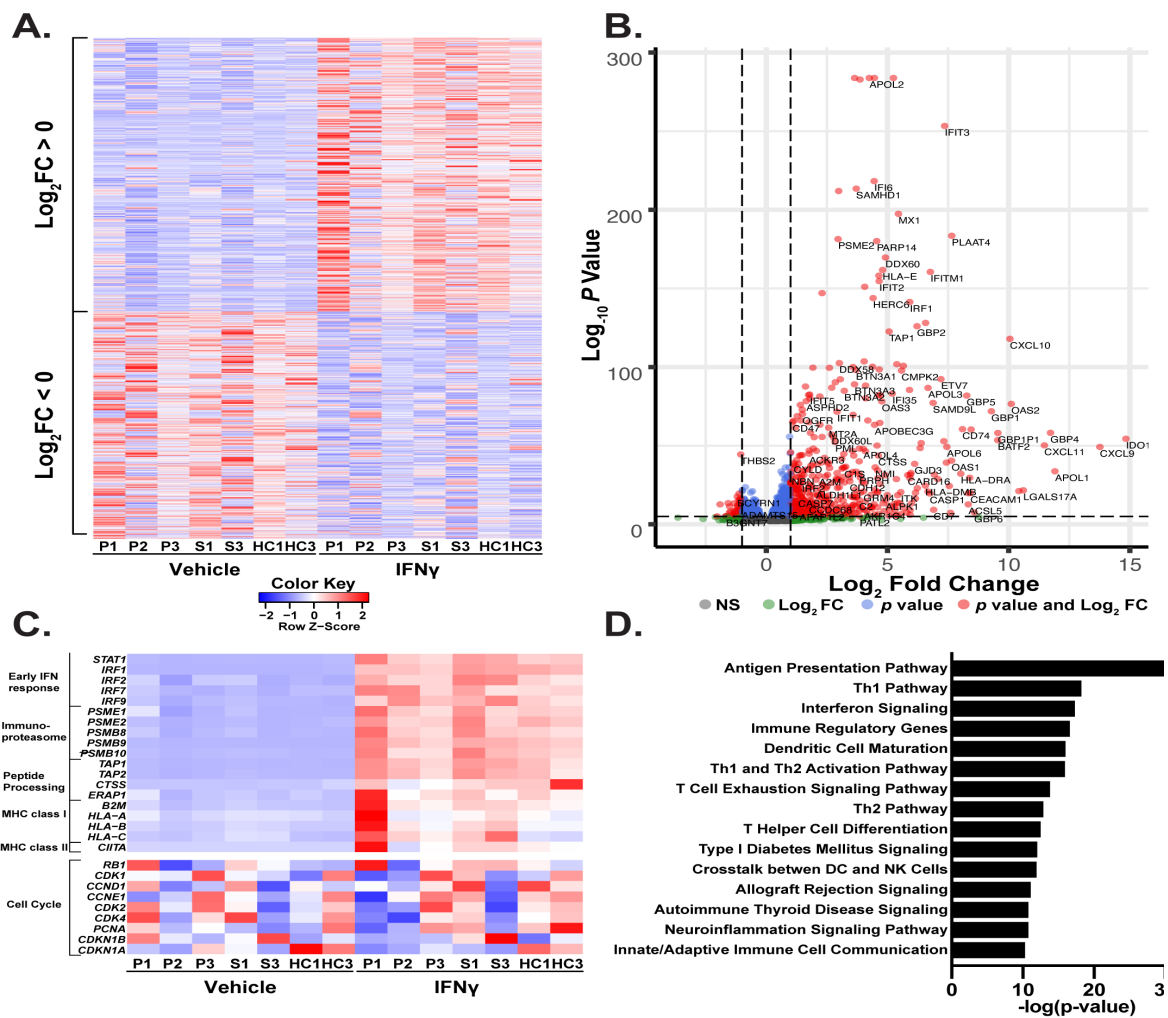


**Figure 12A.** Percentage of cells expressing the astrocyte marker GLAST in untreated cells and cells treated with IFN $\gamma$ , starting at day 36 or 60 of the healthy control group. The group consisted of four donors with one to two experimental replicas each. **12B** shows the average combined expression of GLAST per donor in each group.





**Figure 13.** Figs 13A and B shows the comparison of the percentages of cells expressing O4 and the neuronal marker  $\beta$ III tubulin (TUJ1) for untreated and IFN $\gamma$ -treated cells in the HC group (4 donors, with 2 experimental replicas) starting at day 36 or 60, demonstrating a concomitant increase in TUJ1 to accompany the decrease in O4. Shown are a compilation of data in the graph and representative histograms. Significance was determined with generalized estimating equations with R, and using a p-value less than 0.05.



**Figure 14.** RNA-Seq analysis of IFN $\gamma$  exposure in PDGFR $\alpha$  sorted OPCs. OPCs were sorted for PDGFR $\alpha$  expression from a total of 7 different cell lines generated from the following donors: 3 people with progressive MS (P1-P3), 2 people with mild/stable MS (S1, S3), and two healthy controls (HC1, HC3). After sorting, cells were plated and exposed to 10ng/ $\mu$ L IFN $\gamma$  for 16 hours.

RNA was then isolated and subjected to RNA-Seq analysis as described in the methods section. Fig 5A shows a heatmap of all differentially expressed genes (defined by having an adjusted p value < 0.05) in two sets: those that are upregulated by IFN $\gamma$  (Log<sub>2</sub> Fold Change > 0, n = 1749) on top and those that are downregulated by IFN $\gamma$  (Log<sub>2</sub> Fold Change < 0, n = 1453) on the bottom for each of the seven OPCs tested, showing patterns of similarity and differences between groups and individuals. Fig 5B shows a volcano plot with Log<sub>2</sub> Fold Change on the x- axis and – Log<sub>10</sub> Adjusted p value on the y-axis. Each individual gene is represented by a single dot, and color indicates the following: Red: >2 fold change and adjusted p value < 0.05, Blue: <2 fold change and adjusted p value < 0.05, Green: > 2 fold change and adjusted p value > 0.05, and Grey: < 2 fold change and p value > 0.05. Fig 5C shows a heatmap of IFN $\gamma$  response genes and immune/antigen presentation genes in the top panel, and a subset of cell cycle control genes previously identified<sup>39</sup> in the bottom panel. Fig 5D shows the results of an Ingenuity Pathway Analysis<sup>54</sup> conducted on genes differentially regulated by IFN $\gamma$  (Log<sub>2</sub> Fold Change < -1 or > 1 and adjusted p value < 0.05) for canonical pathway enrichment. Top 15 results are shown, sorted by -Log(p value).

# Conclusion and Future Direction

## 3.1 Summary

iPSCs from six MS donors with varying degrees of disability successfully differentiated to mature oligodendrocytes. Chronic exposure to low-dose IFN $\gamma$  starting at day 36 of differentiation inhibits OPC differentiation as seen by the drastic decrease of O4, MBP, and OLIG2 expression. The inhibitory effects were significantly milder when treatment was delayed to day 60 of differentiation, highlighting a critical period of vulnerability during the early stages. Sustained low-dose IFN $\gamma$  redirects lineage phenotype as seen by the concomitant decreased expression of O4 with increased expression of TUJ1 in the healthy controls. Short-term IFN $\gamma$  exposure on PDGFR $\alpha$  sorted OPCs upregulates immune response pathways and molecules such as MHCs, which aligns with the acquisition of antigen-presenting cell characteristics.

## 3.2 Future direction

Our results shed light on the importance of the environment in which differentiation therapies are administered. The inflammatory milieu recreated in our study showed that the disease environment may profoundly negatively affect the efficacy of the treatment. Therefore, it would be useful for promising remyelinating agents to be tested under these inflammatory conditions, which may provide insights into the individual biology of the patients. Ultimately, decreasing inflammation in the CNS may be necessary for OPC differentiating/remyelinating agents to reach their full efficacy.

# Appendix

## Abbreviations

Bcl-xl.....	B-cell lymphoma-extra large
cDNA.....	Complementary deoxyribonucleic acid
CIITA.....	Class II transactivator
c-Myc.....	Proto-Oncogene transcription factor C-Myc
CNP.....	2',3'-Cyclic-nucleotide 3'-phosphodiesterase
CNS.....	Central nervous system
CXCL#.....	C-X-C Motif Chemokine Ligand
CCL#.....	C-C Motif Chemokine Ligand
DAAM2.....	Dishevelled Associated Activator Of Morphogenesis 2
DAPI.....	.4',6-diamidino-2-phenylindole
EDSS.....	Expanded disability status scale

ET-1.....Astrocyte-derived endothelin-1

Fc.....Fragment crystallizable region

GAPDH.....Glyceraldehyde 3-phosphate dehydrogenase

GLAST (ACSA1).....Glutamate Aspartate Transporter 1 (astrocyte cell surface antigen-1)

GFAP.....Glial fibrillary acidic protein

hESC.....Human embryonic stem cells

HLA.....Human leukocyte antigen

HMGB-1.....High mobility group box 1 protein

IFN $\gamma$ .....Interferon gamma

IL#.....Interleukin

iPSCs.....Induced pluripotent stem cells

IRB.....Institutional review board

IRF#.....Interferon regulatory factor

Klf4.....Kruppel-like factor 4

MBP.....Myelin basic protein

MIF.....Macrophage migration inhibitory factor

MS.....Multiple sclerosis

NF.....Neurofilament

NGS.....Normal goat serum

NKx2.2.....Homeobox protein NKx2.2.

NPCs.....Neural progenitor cells

O4.....Sulfatide O-Antigen 4

Oct3/4.....Octamer-binding transcription factors 3 and 4

OLs.....Oligodendrocytes

OLIG2.....Oligodendrocyte transcription factor 2

OPCs.....Oligodendrocyte progenitor cells

PBMCs.....Peripheral blood mononuclear cells

PCR.....Polymerase chain reaction

PDGFR $\alpha$ .....Platelet-derived growth factor alpha receptor

PPMS.....Primary progressive multiple sclerosis

PSMB#.....Proteasome subunit beta type

PSME#.....Proteasome activator complex subunit

RNA.....Ribonucleic acid

STAT1.....Signal transducer and activator of transcription 1

SOX10.....SRY-Box Transcription Factor 10

TAP#.....Transporter associated with Antigen Processing

TNF $\alpha$ .....Tumor necrosis factor- $\alpha$

TUJ1.....Beta tubulin III

Tx.....Treatment



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# Curriculum Vitae

## Education

08/2015 – 07/2020

Ph.D. (Cellular and Molecular Medicine)  
Johns Hopkins University School of  
Medicine, Baltimore MD.

08/2012 – 05/2015

*Magna cum laude*, Biology with minor in  
Chemistry University of New Mexico,  
Albuquerque NM.

## Research experience

**Johns Hopkins University School of Medicine**, Cellular and Molecular  
Medicine Program

Ph.D.; Thesis Advisor: Dr. Katharine A. Whartenby.

**University of New Mexico School of Medicine**, Cell Biology and Physiology  
Undergraduate Research Assistant; Advisor: Dr. Oscar Bizzozero.

## Publications

**Morales Pantoja IE**, Smith MD, Rajbhandari L, Cheng L, Gao Y, Machairaki V, Venkatesan A, Calabresi PA, Fitzgerald KC, Whartenby KA. iPSCs from people with MS can differentiate into oligodendrocytes in a homeostatic but not an inflammatory milieu. PLOS ONE

<https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0233980>

Chamling X, Kallman A, Berlinick C, Devkota P, Mertz JL, **Morales Pantoja IE**, Smith MD, Chang C, Kaushik A, Chen L, Whartenby KA, Calabresi PA, Mao HQ, Wang TH, and Zack DJ. Single-Cell Transcriptomic Analysis Reveals Molecular Diversity of Human Oligodendrocyte Progenitor Cells. Manuscript under revision.

Glenn JD, **Morales Pantoja IE**, Caturegli MP, Whartenby KA. MyMD1, a novel alkaloid compound, ameliorates the course of experimental autoimmune encephalomyelitis. Journal of Neuroimmunology.

<https://doi.org/10.1016/j.jneuroim.2019.577115>

Hu CL, Nydes M, Shanley KL, **Morales Pantoja IE**, Howard TA, Bizzozero OA. Reduced expression of the ferroptosis inhibitor GPx4 in multiple sclerosis and experimental autoimmune encephalomyelitis. Journal of Neurochemistry.

<https://doi.org/10.1111/jnc.14604>

**Morales Pantoja IE**, Hu CL, Perrone-Bizzozero NI, Zheng J, Bizzozero OA. Nrf2-dysregulation correlates with reduced synthesis and low glutathione levels in experimental autoimmune encephalomyelitis. 139:640-650. 2016. Journal of Neurochemistry. <https://doi.org/10.1111/jnc.13837>

## Awards

Americas Committee for Treatment and Research in Multiple Sclerosis (ACTRIMS) Young Scientist Summit in Clinical Neuroimmunology, January 2020 Poster Award

Biomedical Research Day, University of New Mexico, 2014, 2<sup>nd</sup> place best undergraduate oral presentation award

## Presentations

**Morales Pantoja IE**, Smith MD, Rajbhandari L, Cheng L., Gao Y, Machairaki V, Venkatesan A, Calabresi PA, Fitzgerald KC, Whartenby KA. iPSC-derived Oligodendrocytes from people with MS reveal chronic exposure to IFN $\gamma$  derails differentiation and redirects lineage.

1. Poster presentation delivered at ACTRIMS Forum February 2020, West Palm Beach, FL.
2. Poster presentation delivered at ACTRIMS Young Scientist Summit in Clinical Neuroimmunology January 2020, Austin TX. Poster award.

**Morales Pantoja IE**, Calabresi PA, Whartenby KA, Studying Multiple Sclerosis in a Dish.

1. Oral presentation delivered at Brewing Up Science, Baltimore MD. January 2020

**Morales Pantoja IE**, Marchairaki V, Chamling X, Zack DJ, Calabresi PA, Whartenby KA. Induced pluripotent stem cell-derived retinal ganglion cell model system to study neurodegeneration in progressive multiple sclerosis.

2. Poster presentation delivered at TEDCO 2017 Baltimore, MD.

**Morales Pantoja IE**, Dasgupta A, Zheng J., Bizzozero OA. Protein aggregation inhibitors decrease neural cell death in the spinal cord of diethylmaleate-treated mice.

Oral presentation delivered at Biomedical Research day 2014, University of New Mexico. 2<sup>nd</sup> place best undergraduate oral presentation award.

3. Poster presentation at SACNAS 2014, Los Angeles, CA.
4. Poster presentation at SACNAS 2013, San Antonio, TX.

### **Mentorship**

Training undergraduate students, laboratory technicians, and summer program high school students. 2016–present

### **Research Appointments**

Initiative to Maximize Student Development (IMSD) at the University of New Mexico. 2013-2015

### **Leadership**

Recruitment of talented under-represented minorities at the Annual Biomedical Research Conference for Minority Students (ABRCMS) Phoenix AZ, representing Johns Hopkins.